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Novel, rapid DNA-based on-chip bacterial identification system combining dielectrophoresis and amplification-free fluorescent resonance energy transfer assisted in-situ hybridization (FRET-ISH)

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ABSTRACT

Although real-time PCR (RT-PCR) has become a diagnostic standard for rapid identification of bacterial species, typical methods remain time-intensive due to sample preparation and amplification cycle times. The assay described in this work incorporates on-chip dielectrophoretic capture and concentration of bacterial cells, thermal lysis, cell permeabilization, and nucleic acid denaturation and fluorescence resonance energy transfer assisted in-situ hybridization (FRET-ISH) species identification. Identification is achieved completely on chip in less than thirty minutes from receipt of sample compared to multiple hours required by traditional RT-PCR and its requisite sample preparation.

Keywords: fluorescence resonance energy transfer assisted in-situ hybridization (FRET-ISH), in-situ hybridization (ISH), dielectrophoresis (DEP), on-chip diagnostics, microbial identification

1. INTRODUCTION

Prompt public health investigation and response necessitates rapid identification of low bacterial concentrations. Although established as a gold standard for nucleic acid based diagnostics, most real-time PCR (RT-PCR) approaches remain time-intensive due to sample preparation and amplification cycle times. Presented here is a novel DNA-based diagnostic assay combining dielectrophoretic bacterial capture and concentration, on-chip thermal lysis, cell permeabilization and nucleic acid denaturation with fluorescence resonance energy transfer assisted in-situ hybridization (FRET-ISH). This platform facilitates nucleic acid detection in approximately thirty minutes from receipt of sample (Table 1), compared to multiple hours required by traditional RT-PCR and its requisite sample preparation.

Fluorescent in-situ hybridization (FISH) was first introduced in the 1980s and has since found widespread application in bacterial identification [1-5]. Although allowing species–specific microbiological detection, FISH traditionally requires fixation, permeabilization, denaturation, probe hybridization, washing, and detection. Together, the complete process can take greater than twenty-four hours and is often plagued by inadequate sensitivity and specificity [1]. Adaptation of FISH techniques with microfluidic sample preparation steps and fluorescence resonance energy transfer (FRET)-based detection dramatically decreases assay time while increasing both sensitivity and specificity [2, 6-9].

Dielectrophoresis (DEP) offers a simple and near-instantaneous mechanism for bacterial capture and concentration from small diluted sample volumes. DEP forces arise from the interaction of gradients in non-uniform high frequency (AC) electric fields with dipole moments that are induced in polarizable particles. The sign and magnitude of the forces can be estimated from calculating the real part of the frequency-dependent Clausius-Mossotti factor ($Re[F_{CM}]$), which depends on the relative conductivities and permeabilities of the medium and the particle [10]. In positive dielectrophoresis (pDEP), $Re[F_{CM}]$ is greater than 0 and the particle moves up the gradient toward locations of greatest electric field (typically at the edges of electrodes), whereas in negative dielectrophoresis (nDEP), $Re[F_{CM}]$ is less than 0 and the

particle is repelled from locations of greatest electric field [11]. The device operating frequency is selected to provide the desired DEP regime. The present method therefore imposes fields at approximately 1 MHz to ensure efficient pDEP capture and concentration.

After isolation, cell lysis is a required step for most nucleic acid-based assays [12]. Both off-chip and on-chip methods of lysis have been employed for downstream microfluidic molecular detection of bacteria, including ultrasonic, physical disruption, temperature, and chemical lysis [13-15]. Although often utilized, chemical lysis techniques remain time-consuming and complex due to subsequent purification steps to prevent interference with detectable molecules or assay processes. Alternatively, thermal lysis uniquely integrates cellular permeabilization and nucleic acid denaturation, often without additional purification requirements.

FRET procedures function on the basic concept of energy transfer between two dyes, a high energy donor and a low energy acceptor at a certain transfer rate, K_T [16]. FRET efficiency (E) is dependent on the ability of the donor to transfer its internal energy to the acceptor, which can be calculated as a function of donor dye decay time changes due to the presence of an acceptor dye. In the presented methodology, the DNA of intact bacterial cells is first stained with Invitrogen ® SYTO®-9 bound to the minor groove. Then, only when a second, enterobacterial-specific 6-Carboxy-2',4,4',5',7,7'-hexachlorofluorescein (HEX)-labeled probe is bound to the complementary DNA will a minimum critical distance necessary for energy transfer be achieved. Use of this relative method for FRET-ISH detection minimizes the influence of non-specific signals often seen in traditional FISH assays.

Table 1. FRET-ISH assay times

Bacterial centrifugation and preparation	6 min
Sample delivery to chip	1 min
Dielectrophoretic capture and concentration	1 min
Cell lysis, permeabilization and nucleic acid denaturation	5 min
Nucleic acid hybridization	5 min
Detection and data analysis	5 min
Total Time	**23 min**

2. MATERIALS AND METHODS

2.1 Fluorescent staining of cells

Escherichia coli C3000 (ATCC Cat. No. 15597) was centrifuged at 5000 rpm for five minutes at room temperature and resuspended in filtered, distilled water prior to analysis. Then, prior to chip delivery, intracellular bacterial DNA was labeled with Invitrogen® SYTO®-9 fluorescent nucleic acid stain (Ex. 488 nm, Em. 500 nm) to monitor dielectrophoretic capture concentration of bacteria [17]. Additionally, the SYTO®-9 stain was utilized as donor dye for the downstream specific FRET-ISH assay. Stained bacteria was then diluted in 10 mL distilled water for a final concentration of 1.23×10^6 cells/mL. HEX-tagged enterobacterial repetitive intergenic consensus (ERIC) probe (5′-ATGTAAGCTCCTGGGGATTCAC-3′, T_m=54.8 ^oC, 11.1 ng/mL) from Integrated DNA Technologies® (Ex. 532 nm, Em. 560 nm) was added to the bacterial solution immediately prior to dielectrophoretic capture [2].

2.2 Dielectrophoretic capture and concentration of cells

Dielectrophoresis was performed inside silicon-and-glass chips fabricated using standard cleanroom microfabrication techniques. In brief, a 4" silicon wafer was first wet-oxidized to form a 200 nm SiO_2 insulating layer, on top of which 250 nm of Cr-Au metal was sputter-deposited. The metal was patterned by standard photolithography and wet-etching (AZ 1518 resist, Transene gold etch TFA, Cyantek CR-7 chrome etchant). A second 4" wafer made of borosilicate glass was drilled with 500 μm diameter through-holes (Bullen Ultrasonics) to provide fluid access ports. After drilling, a Cr-Au metal layer was sputter-deposited to serve as a mask for fluid channel etching. The fluid channel pattern was wet-etched in the metal mask, then the glass was etched to a depth of 10-15 μm using a solution of 22% hydrofluoric acid and 78% acetic acid. After stripping the metal etch-mask, the glass and silicon chips were anodically bonded together (constant voltage -900 V for ~5 min.) at 350 °C to form the sealed fluid channels 2.6 mm wide and 60 mm long.

Interdigitated electrodes in the chips (Figure 1) were 40 µm wide with 40 µm spacing. Individual chips were diced apart and wire leads were attached to electrode contact pads with silver paint and epoxy.

The mixture of SYTO®-9-stained bacteria and HEX-labeled ERIC probe solution in diH_2O was introduced on chip by a syringe pump at a fixed rate of 100 µL/min for one minute. Cells were dielectrophoretically captured and concentrated at a frequency of 1 MHz and voltage of 10 V_{p-p} modulated by a standard digital function and waveform generator (Agilent 33220 A).

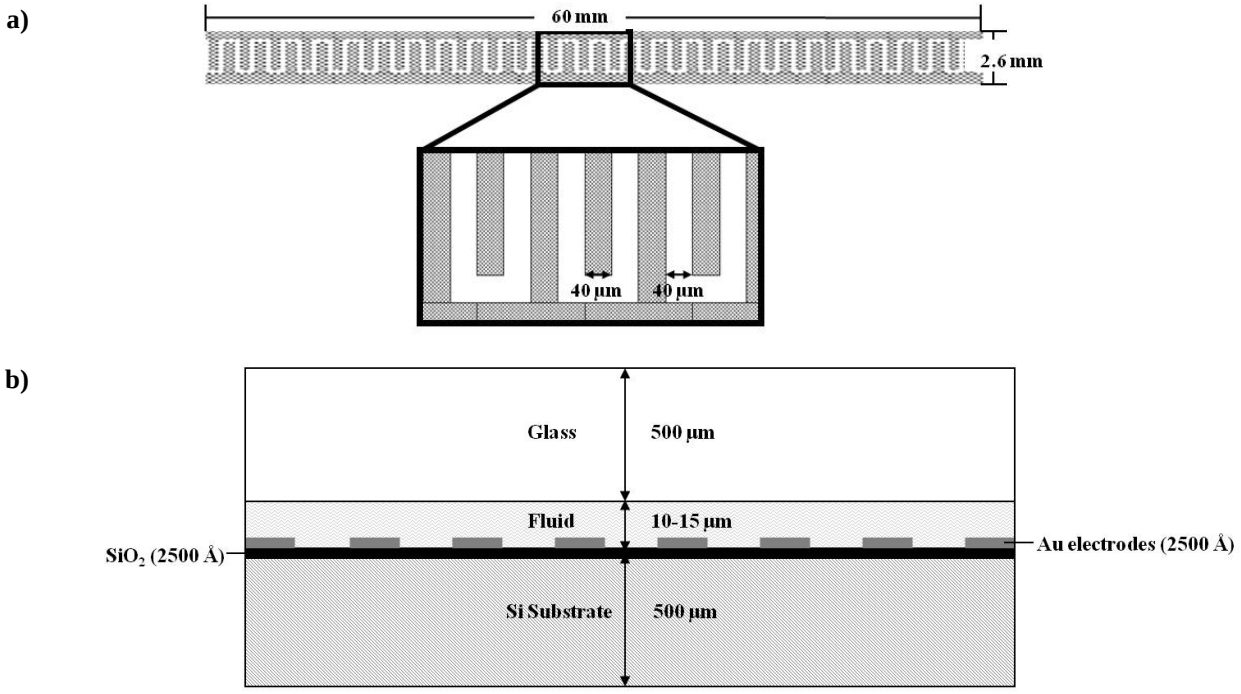

Figure 1. Dielectrophoresis chip design a) top view and b) cross-sectional view.

2.3 Thermal lysis, permeabilization and nucleic acid denaturation and hybridization

Cells were lysed and permeabilized, and nucleic acids were denatured on-chip at 65°C for five minutes by a Kapton® KHLV series (Polyimide Film and FEP adhesive) rectangular insulated heater (28 Volts, 0.5x2 in, 10 watts per in^2) adjacent to the chip and modulated with a thermocouple-attached temperature controller (Alpha Omega Instruments Series 800).

2.5 Imaging and data analysis

To assess effectiveness of capture and concentration, time-lapse images were acquired at a rate of 1 frame/s on a Zeiss Axiovert 5100 filter-based fluorescent microscope. Samples were identically excited with a 485/20 nm filter, and emission was detected at 505/10 nm . FRET-ISH efficiency was determined by quantification of donor dye photobleaching. Decay times were calculated for SYTO®-9 stained bacterial samples unbound and bound to the HEX-labeled ERIC probe. The assay transfer rate, k_T and FRET efficiency, E were calculated experimentally as $k_T=(1/T_{DA})-(1/T_D)$ and $E= 1-(T_{DA}/T_D)$ where T_D = fluorescence lifetime of donor without acceptor and T_{DA}= fluorescence lifetime of donor in the presence of acceptor. All images were acquired with a ScopeTek 2.0M pixel CCD camera and MiniSee software. Fluorescent signal was analyzed with ImageJ software to quantify increase in signal from labeled cells indicative of cell concentration and capture [18]. Quantitative analysis and curve fitting was conducted in Microsoft Excel.

3. RESULTS AND DISCUSSION

3.1 Dielectrophoretic capture and concentration of cells

Whereas the initial on-chip cell population was only slightly detectable (Figure 2a), bacterial presence at electrodes after concentration is evident and easily discernable (Figure 2b). At a rate of 100 uL/min for one minute, bacteria was successfully captured and concentrated greater than 400 times by dielectrophoresis (Figure 3). Increase in bacterial concentration as measured by SYTO®-9 signal was linearly correlated over time ($y = 8.2699x - 43.106$, $R^2 = 0.9862$).

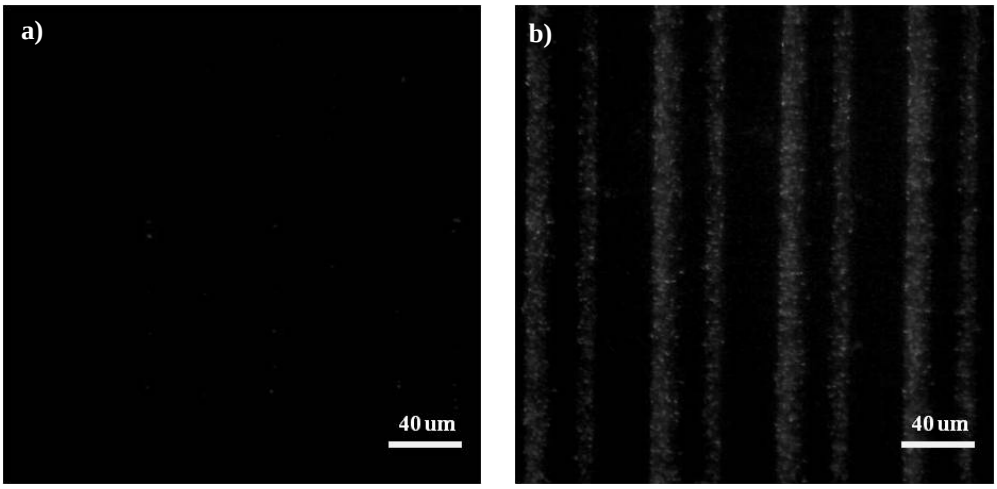

Figure 2. Dielectrophoretic capture and concentration of bacterial cells. (a) Prior to dielectrophoretic capture and concentration, detectable SYTO®-9 stained bacteria are limited and sparsely distributed. (b) After one minute of capture at 1 MHz and 100 uL/min, bacteria is evident and expressing a signal over 400X greater than initially recorded.

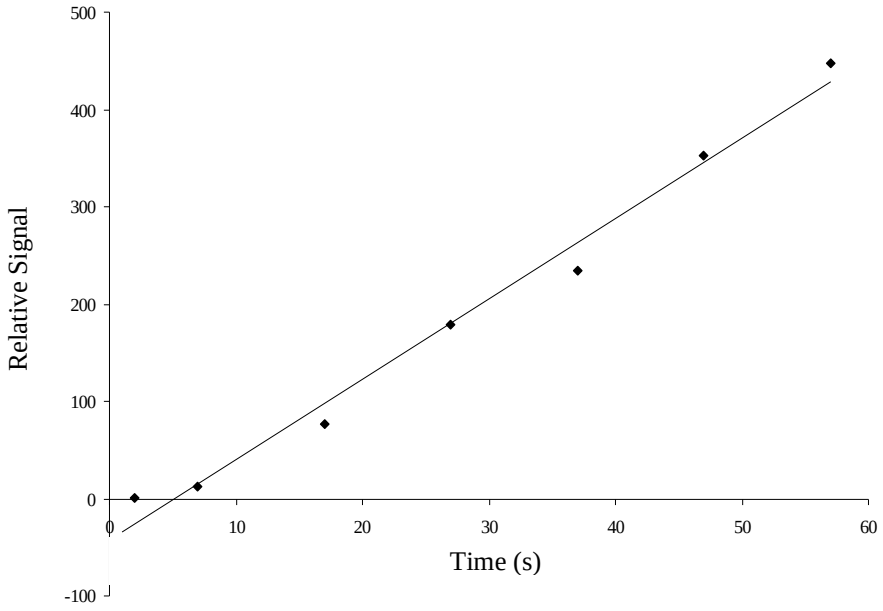

Figure 3. Dielectrophoretic bacterial concentration. Signal from SYTO®-9 labeled bacteria increased linearly over time, measuring more than 400X the initial value after one minute flow at 100 uL/min.

3.3. Probe-Based Identification: FRET-ISH

A regression line fitted to relative intensity over time found decay time of donor dye alone (T_D) and in the presence of the HEX-labeled ERIC probe (T_{DA}) to be 212.95 s and 87.91 s, respectively (Table 2). FRET-ISH efficiency was determined by quantification of donor dye photobleaching. Decay times of emission at 505/10 nm were calculated for SYTO®-9 stained bacterial samples unbound and bound to the HEX-labeled ERIC probe. The assay transfer rate, k_T and FRET efficiency, E, were then determined to be 6.68 ns^{-1} and 58.7%, respectively (Table 2), indicative of exceptional probe binding within nanoscale proximity of the SYTO®-9 dye.

Table 2. FRET-ISH decay times, transfer rate and FRET efficiency

SYTO®-9 alone decay time (T_D)	212.95 s
SYTO®-9 with bound probe decay time (T_{DA})	87.91 s
Transfer rate (K_T)	6.68 ns^{-1}
FRET Efficiency (E)	58.7%

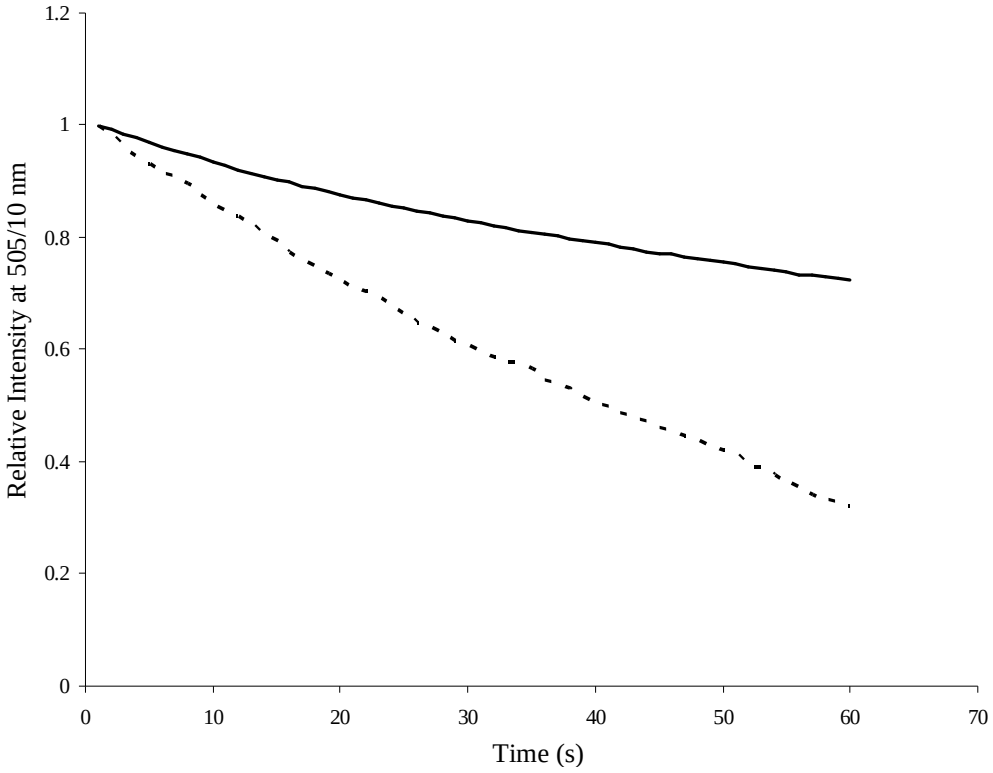

Figure 4. Fluorescent decay curves. Photobleaching of donor without acceptor (——) and donor in presence of acceptor (- - -) when excited at 485/20 nm for sixty seconds.

4. CONCLUSIONS

Application of the FRET-ISH assay for bacterial detection and identification is a reliable and rapid alternative to traditional RT-PCR. As with other DNA-based tests, FRET-ISH can be easily applied to a variety of bacteria for which DNA probes are available. Although photobleaching by filter-based microscopy lacks the precision and accuracy of

laser-driven lifetime imaging, the relational nature of the decay time calculations minimizes these concerns and through probe binding, provides significant confirmation of bacterial presence. For scarce bacterial concentrations, dielectrophoretic concentration times can be increased without significant contribution to assay time. In addition, for populations where capture efficiency is especially sensitive, flow rates can be decreased to maximize percent captured. Finally, by integrating rapid sample concentration and detection with minimal equipment, the FRET-ISH assay shows great promise for future field applications.

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